Immunoregulation in Rheumatic Fever*

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SUMMARY T-cell subsets defined by monoclonal antibodies, *in vitro* immunoglobulin biosynthesis, lymphoproliferative response to phytohaemagglutinin (PHA), and autologous mixed lymphocyte reaction (AMLR) were studied in 19 childrens with acute rheumatic fever (ARF), 14 of those 19 who were available for follow-up after recovery, 14 with uncomplicated streptococcal pharyngitis, 18 with post-streptococcal acute glomerulonephritis, and 30 normal people. The results showed: 1) the ARF patients had much lower mean percentages and absolute numbers of active T, total T, OKT3 and OKT8 cells when compared with normal subjects, 2) the blastogenic response to PHA and AMLR was impaired in ARF patients, and 3) in the *in vitro* immunoglobulin biosynthesis study, the B lymphocytes from the ARF patients produced a much greater amount of IgG than did those from normal subjects and the overproduction was due to a deficiency of suppressor cell activity in the patients' T-cell population. Thus, the altered T-cell functions, especially the loss of the suppressor T-cell subset both in number and function, may explain partly the pathologically augmented humoral immune response in ARF patients.

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Although a large body of circumstantial evidence suggests that streptococcal infections of the upper respiratory tract are causally related to acute rheumatic fever (ARF) and its residue as chronic rheumatic heart disease (RHD), the precise nature of the link continues to puzzle the investigators. Despite the finding that there is cross antigenicity between human heart muscle antigens and streptococcal antigens,[1-5] several lines of evidence strongly suggest that the unusually heightened humoral and cell-mediated immune responses may play an important role in the pathogenesis of those nonsuppurative sequelae of the streptococcal infections: 1) the rheumatic patients had higher antistreptolysin 0 titres than did the patients with uncomplicated streptococcal infections,[6] 2) the titres of heart muscle antibody of the rheumatic patients were much higher than those of nonrheumatics,[7,8] 3) the autoantibodies against subthalamic and caudate nuclei occurred more frequently in ARF patients either with (46.6%) or without (14.0%) chorea than in normal subjects and other patients with streptococcal infections,[9] 4) there was a clear relationship between the migration inhibition test to streptococcal cell wall products and ARF,[10] 5) the rheumatic patients showed more intense delayed-type skin hypersensitivity to streptococcal antigens than did the nonrheumatics,[6,11] 6) the lymphocytes from the rheumatic patients synthesised much more DNA after *in vitro* stimulation with streptococcal[12] and heart muscle antigens[13] than did those from normal subjects, and 7) the infiltrating lymphocytes in both the fresh and chronic rheumatic valvular tissues belonged to T-cell lineage with the helper (OKT4) T-cell phenotype predominating.[14]

Since a delicate balance between helper and suppressor (regulatory) T cells is required to ensure an appropriate immune response, it is logical to suppose that the enhanced immune response to streptococcal, heart muscle, and subthalamic and caudate nuclei antigens in rheumatic patients may be due to a specific deficiency in the regulatory T-cell function during the acute phase of rheumatic fever.

This project was conducted to examine the T-cell functions in rheumatic patients by studying: 1) the distribution of T-cell subsets defined by monoclonal antibodies,[15] 2) the autologous mixed lymphocyte reaction which is considered to be an *in vitro* phenomenon reflecting the *in vivo* immunological communication between T cells and self non-T-cells,[16,17] and 3) the regulatory function of T cells

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of ARF patients in *in vitro* immunoglobulin biosynthesis.

MATERIALS AND METHODS

Study populations included 19 children with ARF. The diagnosis of ARF was based on the revised Jones criteria[18] and all the patients had carditis at the time of admission. The majority (17 out of 19) were studied within one week after the onset of the disease. None had received steroids and only a few had taken one or two doses of aspirin at the time of blood sampling. Also included in the study populations were 14 out of the 19 children with ARF who had recovered from the acute attacks for at least six months and who were on long-term penicillin prophylaxis. In addition, 14 children with acute streptococcal pharyngitis which had been proven by clinical pictures, positive culture and/or elevated antiştreptolysin 0 (ASLO) titres were included as was a population of 18 children with post-streptococcal acute glomerulonephritis diagnosed by the combination of recent upper respiratory infection, clinical pictures of nephritis, decreased serum complement levels and elevated ASLO titres. The final population included 30 healthy individuals ranging in age from eight to 23 years.

Purification of mononuclear cells (MNCs) lymphocytes and monocytes

Peripheral MNCs were prepared by Boyum's method.[19] After washing three times with Hank's balanced salt solution (HBSS, Gibco), the MNCs were suspended at a concentration of $2x10^6$ cells/ml in complete culture medium (RPMI-1640 containing 10% heated foetal calf serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 2 mM/ml of L-glutamine, (Gibco)). Fifteen ml of cell suspension were placed into a plastic petri dish (100x15 mm, Falcon Plastics) and incubated in a 37°C, 5% CO_2, humidified incubator for one hour. At the end of the incubation period, the non-adherent cells (referred to as pure lymphocytes, which contained 66-78% E-rosette-forming cells, 19-28% EAC-rosette-forming cells and less than 2% peroxidase-positive cells) were decanted, washed three times with HBSS and resuspended in HBSS at a concentration of $2x10^6$ cells/ml. Ten millilitres of cold HBSS were added to the petri dish and incubated at 4°C for 10 minutes. The adherent cells which contained more than 95% peroxidase-positive cells (monocytes) were harvested using rubber policemen; they were washed and resuspended at a concentration of $1x10^6$ cells/ml in complete culture medium.

Separations of T and B lymphocytes were done by mixing equal volumes of pure lymphocyte suspension and neuraminidase-treated sheep RBC suspension, centrifuged at 200xG at room temperature for five minutes and incubated in ice water for one hour. The pellet was resuspended very gently, layered over Ficoll/Hypaque gradient and centrifuged at 400xG at room temperature for 30 minutes. The cells on the interface layer were aspirated and washed; they were referred to as the B-cell-enriched population, which contained less than 10% peroxidase-positive cells and less than 5% E-rosette-forming cells. The T cells forming E-rosettes were pelletted to the bottom and were freed from SRBC by hypotonic lysis with cold distilled water. Over 90 per cent of the T cells prepared in this way could reform E-rosettes and about 88 per cent of them could be stained by FITC-conjugated anti-OKT3 (Orthoclone, Raritan, NJ). Both the T-cell and B-cell enriched populations were resuspended in complete culture medium at a concentration of $2x10^6$ cells/ml.

Determinations of lymphocyte subpopulations

Total T, active T and B cells (EAC-rosettes) were counted by the method of Kerman *et al*[20] with slight modification.[21] Surface immunoglobulin-bearing B cells were detected by the direct immunofluorescence techinque, using fluorescein-conjugated rabbit anti-human $F(ab')_2$ (Cappel, U.S.A.). Enumerations of T-cell subsets defined by monoclonal antibodies (OKT series, Orthoclone) were done by the method described previously.[22] Anti-OKT3 defined total T cells (OKT3 cells); anti-OKT4, T cells with helper/inducer functions (OKT4 cells); and anti-OKT8, T cells with cytotoxic/suppressor properties (OKT8 cells).[15]

The autologous mixed lymphocyte reactions (AMLR) were carried out by the method reported previously.[23] One-tenth ml of T-cell suspension was mixed with an equal volume of B-cell suspension and then cultured in triplicate in round-bottom microtitre plates (Flow Laboratories) for seven days in a 37°C, 5% CO_2, humidified incubator. The B cells were pretreated with 50 μg/ml of mitomycin C for 30 minutes at 37°C. Twenty hours before the end of culture, 1μCi ^{3}H-thymidine (^{3}H-TdR, New England Nuclear, 6.7 Ci/mM) in 20 μl RPMI-1640 was added to the cultures. The cells were harvested by an automatic harvester (Microharvester, Bellco) and the radioactivity counted. The stimulation index was calculated by dividing the mean cpm of stimulated cultures by that of their unstimulated counterparts.

The lymphoproliferative responses to phytohaemagglutinin (PHA) were done by the Bradley's method.[24] One-tenth ml of MNCs in a concentration of $1.5x10^6$ cells/ml of complete culture medium was added simultaneously with 0.1 ml of RPMI-1640 diluted PHA-M (final dilution 50x, Gibco) to the round-bottom, multiple-well microtitre plates. The cultures were incubated for three days in a 37°C, 5% CO_2, humidified incubator. At the end of culture, 20 μl of RPMI-1640 containing 1 μCi of ^{3}H-TdR were

Table 1 Distributions of lymphocyte subpopulations

Subjects	Lymphocytes (/cumm)	Active T	Total T	B		OKT3	OKT4	OKT8
				EAC-rosettes	sIg			
Active RF (n = 19)	4,286±488*	16.4± 6.5**† (703±277)*††	54.7±12.6☆ (2,344±540)★	21.6±6.2 (926±266)	10.7±3.8 (458±163)	64.8±10.1○ (2,777±433)●	45.0±9.5 (1,928±407)	29.4±6.0 □ (1,260±257)△
Inactive RHD (n = 14)	3,838±414	23.2± 7.6 (890±292)	62.8± 7.9 (2,410±303)	20.4±3.9 (783±150)	11.4±3.7 (436±142)	71.3± 9.6 (2,736±368)	43.2±4.1 (1,658±157)	34.8±5.6 (1,336±215)
Streptococcal pharyngitis (n = 14)	4,648±609	24.1± 8.2 (1,120±381)	66.4± 9.8 (3,086±456)	23.9±4.2 (1,111±195)		69.5± 8.1 (3,230±376)	43.9±7.5 (2,040±349)	34.2±5.7 (1,590±265)
Poststreptococcal AGN (n = 18)	4,519±632	23.4± 7.6 (1,057±343)	63.2±10.2 (2,856±461)	24.5±3.0 (1,107±136)		70.6± 9.8 (3,190±443)	43.8±5.8 (1,979±262)	34.6±5.5 (1,564±249)
Normal subjects (n = 30)	3,984±475	24.5±10.5† (976±418)††	65.8±10.9☆ (2,621±434)★	22.3±4.3 (888±171)	10.5±2.9 (418±116)	72.4± 6.8○ (2,884±271)●	46.1±4.5 (1,837±179)	35.1±5.0□ (1,398±199)△

* Absolute number (Mean ± SD), ** Percent (Mean ± SD), † $P < 0.003$, †† $P < 0.008$, ☆ $P < 0.001$, ★ $P < 0.03$, ○ $P < 0.002$, ● $P > 0.1$, □ $P < 0.001$, △ $P < 0.02$.

added. The cells were harvested the next morning and the radioactivity counted. Both the controls and stimulated cultures were set up in triplicates. The stimulation index was calculated by the same method used in AMLR.

In vitro immunoglobulin biosynthesis.

The co-cultivation technique of Waldmann *et al*[25] was used to study the regulatory function of T cells. MNCs from the patients and normal subjects were cultured alone or co-cultivated. MNCs were further fractionated into B cells, T cells and monocytes as previously described. One volume of B cells was mixed with four volumes of T cells and cultured in the presence of 5% monocyte and 10 μl pokeweed mitogen (Gibco) per ml of culture medium in a 37°C, 5% CO_2, humidified incubator for one week. At the end of the culture, the supernatants were collected by centrifugation and the immunoglobulin G (IgG) concentrations in the supernatants were determined by the solid-phase immunofluorescence assay (Immuno-fluor, Bio-Rad Laboratories, Richmond, California).

Statistics

The Student's t test was used for statistical analysis throughout the study.

Table 2 Lymphoproliferative responses to phytohaemagglutinin

Subjects	No. of case	Unstimulated	Stimulated	Stimulation index
Normal subjects	30	984±264*	102,489±20,412	92.4±14.0†☆
ARF patients	19	1,442±331	72,351±18,225	52.3± 7.9☆
Inactive RHD	14	1,028±310	110,072±22,431	98.2± 9.3
Streptococcal pharyngitis	14	1,248±408	120,494±21,544	98.1±11.4
Poststreptococcal AGN	18	1,329±384	112,346±23,149	88.2± 9.5

*Mean ± SEM (counts per minute), † Mean ± SEM, ☆ $P < 0.02$

RESULTS

The distribution of lymphocyte subpopulations in all the groups studied is shown in Table 1. Both the mean percentages and absolute numbers of active T, total T, OKT3 and OKT8 cells of ARF patients were significantly lower than those of the normal subjects, but all these values returned to normal six months after the patients had recovered from acute attacks. There was no difference between the lymphocyte subpopulations of patients with uncomplicated streptococcal pharyngitis and post-streptococcal AGN, and the normal subjects.

The results of lymphoproliferative response to PHA are shown in Table 2. It is clear that only the stimulation index for ARF patients was significantly lower than that for the normal subjects (52.3 ± 7.9 vs 92.4 ± 14.0, $p < 0.02$) and it was due to higher background cpm and lower cpm of the stimulated cultures (although both were not significantly different from that of the normal subjects). The mitotic capability of lymphocytes of ARF patients returned to normal six months after their recovery from acute attacks.

The proliferative responses in terms of the stimulation index of T cells to autologous B cells in various groups studied are presented in Figure 1. While the mean stimulation index of ARF patients was much lower than that of normal subjects (2.9 ± 0.9 vs 8.8 ± 2.2 [mean ± SEM], $p < 0.02$), no such difference was found for other groups of patients when compared with normal subjects. Furthermore, the impaired proliferative ca-

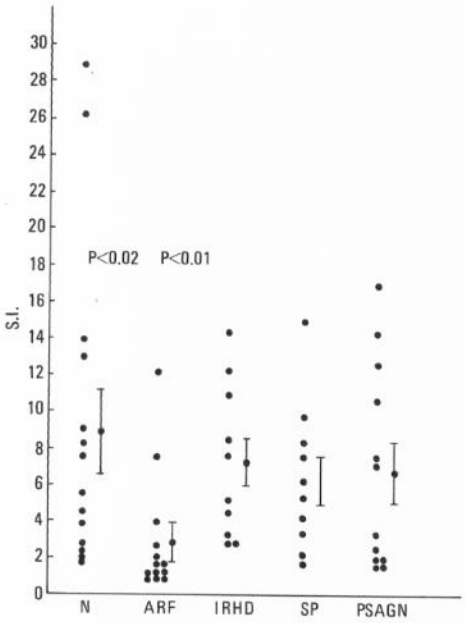

Fig. 1 The results of autologous mixed lymphocyte reaction in 15 normal subjects (8.8 ± 2.2, Mean ± SEM), 13 ARF (2.9 ± 0.9), 10 inactive RHD (IRHD, 7.2 ± 1.3), 10 streptococcal pharyngitis (SP, 6.3 ± 1.2), and 12 poststreptococcal acute glomerulonephritis (PSAGN, 6.6 ± 1.6) patients. Only the stimulation index of ARF was significantly lower than that of normal subjects ($p < 0.02$). After recovery from ARF, AMLR also returned to normal ($p < 0.01$).

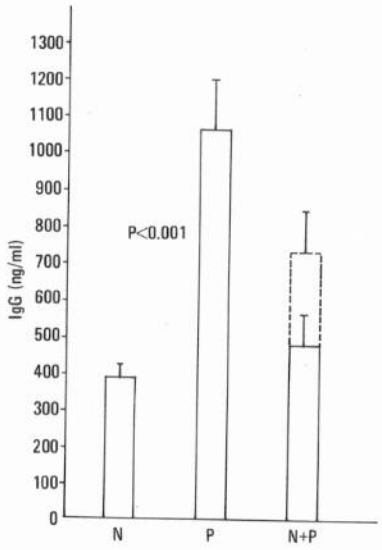

Fig. 2 In vitro IgG biosynthesis. Peripheral blood mononuclear cells (MNCs) from six patients with ARF and six normal subjects were cultured alone or co-cultivated. The patients' MNCs produced a much greater amount of IgG than did those from normal subjects (1,059 ± 137 ng/ml vs 399 ± 37 ng/ml, Mean ± SEM, $p < 0.001$). The IgG synthesised by patients' MNCs was reduced by an average of 34.3 ± 19.0% ($p < 0.05$, dotted bar) after incubation with normal MNCs.

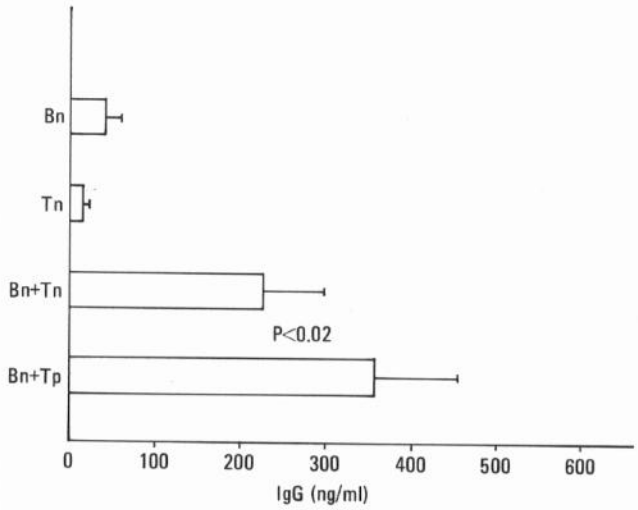

Fig. 3 The effect of patients' T cells on the *in vitro* IgG synthesis of normal B cells. The IgG synthesised by normal B cells (Bn) was much increased when they were cultured with patients' T cells (Tp) instead of self T cells (Tn).

pability of T cells to autologous B cells in ARF patients recovered six months after an acute attack.

As shown in Figure 2, the MNCs from ARF patients produced a much greater amount of IgG than did those from normal subjects (1,059 ± 137 ng/ml vs 339 ± 37 ng/ml, $p < 0.001$) and the synthesis of IgG by the patients MNCs was reduced by an average of 34.3 per cent when incubated with MNCs from normal subjects. Furthermore, while T cells from the patients were able to enhance the IgG production by B cells from normal subjects (Fig. 3), T cells from normal subjects were capable of suppressing the IgG synthesis by B cells from the patients (Fig. 4). Taken together, all these data suggested that the augmented antibody production by patients' B cells might be due to a deficit of regulatory T cells.

DISCUSSION

Although the causal relationship between streptococcal infection and rheumatic fever has been generally accepted, the underlying immunopathogenesis for the occurrence of rheumatic fever remains to be delineated. The majority of investigators agree that the humoral immune responses to streptococcal, human heart muscle, and subthalamic and caudate nuclei antigens are pathologically heightened in patients with ARF,[6-9] but controversy still exists regarding the antigen-specific cell-mediated immunity in those individuals. While some authors reported enhanced sensitivity of lymphocytes from ARF patients to streptococcal and heart muscle antigens,[6,10-13] other investigators presented contradictory results.[26-30] As T cells play a crucial role in maintaining the immune homeostasis, it is mandatory to re-evaluate, by applying newly developed immunological techniques, the T-cell functions in patients with ARF to disclose the underlying immunopathogenesis of rheumatic

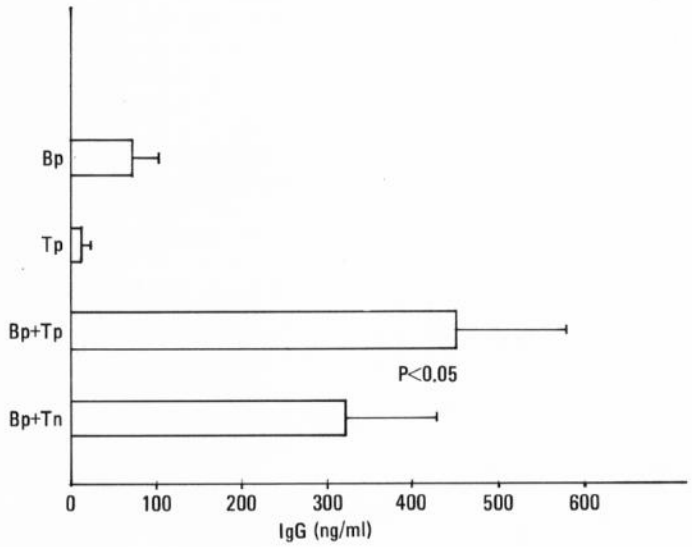

Fig. 4 The effect of normal T cells on the *in vitro* IgG synthesis of patients' B cells. The IgG synthesised by patients' B cells (Bp) was much reduced when they were cultured with normal T cells (Tn) instead of self T cells (Tp).

fever and its sequelae.

The finding in this study that the mean percentage of total T cells detected in ARF patients by the E-rosetting technique was lower than that of normal subjects was in line with those reported by Sapru *et al*[12] and Leuker *et al.*[31] Moreover, the same result was obtained when total T cells were counted by fluorescence-conjugated antibodies against T-cell surface antigens, no matter whether heteroantisera[31] or monoclonal antibody such as the anti-OKT3 in this study were used. The lower mean percentage of active T cells in ARF patients observed in this series was in contrast to that reported by Williams *et al.*[32] The reasons for this discrepancy might be due largely to the different patient population selected for study: 1) in our study, all children had acute carditis, whereas in their study only 55 per cent (22/40) had carditis as the major presenting clinical picture (although a larger proportion already showed physical signs of mitral stenosis and/or mitral insufficiency), 2) while none of our cases had received steroids and only a few took the first or second dose of aspirin, the majority of their cases were being treated with salicylates and three children received 5-10 mg of prednisolone daily and 3) the majority of our patients (17/19) were studied within one week after the onset of ARF, but their patients were studied at a much later time, i.e. one to four weeks after the onset of initial symptoms. As active T cells have been shown to reflect closely the cellular immune status and are actively involved in various facets of cell-mediated immunity,[33,34] the decreased numbers of both active and total T cells found in this study provide an evidence of defective T-cell function in patients with ARF. Moreover, such a result may be used to account for the decreased reactivity of lymphocytes in mixed lymphocyte culture from patients with ARF.[35]

The PHA-induced lymphoproliferative response was impaired in ARF but was normal in inactive RHD patients and others recently infected with *Streptococci.* The result was consistent with those of Sapru *et al*[12] and Meric *et al,*[13] who reported a decreased PHA response in ARF patients, and with those of Francis *et al*[27] and Gray *et al,*[30] who reported a normal PHA response in cases of inactive RDH. It is interesting to note that in patients with ARF the PHA response decreased in the face of normal OKT4 cells although it is the OKT4 cells and not the OKT8 cells which are responsible for the majority of PHA proliferation.[15] Meric *et al*[13] reported that 10 out of 13 plasma samples from patients with ARF were able to cause 50 per cent or more inhibition in the *in vitro* PHA response of lymphocytes in the healthy controls. This inhibitory plasma factor together with the decreased active T cells may be used to explain the discrepancy between the number and function of OKT4 cells in ARF patients. However, as OKT4 cells are heterogeneous,[36-38] it may be worthwhile to use other monoclonal antibodies to study the OKT4 subpopulations in patients with ARF.

Reinherz *et al*[15] have shown that T cells carrying OKT4 antigen provide help for B cell differentiation and immunoglobulin secretion and maturation of effector functions of other T cells; to the contrary, those T cells carrying OKT8 antigen suppress the afore-mentioned biological phenomena. We therefore studied the distributions of OKT4 and OKT8 cells in rheumatic children to see whether there is an imbalance between these two T-cell subsets. It is interesting to find that the mean percentage and absolute number of OKT8 cells was much lower in patients with ARF when compared to that of normal subjects, but no such difference was found among patients with other diseases and normal subjects. This result is consistent with that reported by Williams *et al.*[39] An *in vitro* immunoglobulin biosynthesis study was performed to study further the regulatory function of T cells in ARF patients. The data obtained indicated that the B cells from ARF patients produced a much greater amount of IgG than did those from normal subjects and that the overproduction was due to a deficiency of suppressor cell acti-

vity in the patients' T-cell population. Taken together, the deficiency of OKT8 cells, both in number and function, in the peripheral blood of ARF patient is of particular interest in view of the report by Raizada *et al*[14] that OKT4 cells predominated in the lymphocytic infiltrates in fresh and chronic rheumatic valvular tissues, as the loss of suppressor (OKT8) cells will facilitate, in addition to hyperreactivity of B cells, the occurrence of (OKT4) cell-mediated inflammatory process and finally will result in the tissue damage seen in ARF.

The *in vitro* proliferation of T cells in response to stimulation by autologous non-T cells has been called autologous mixed lymphocyte reaction (AMLR). This *in vitro* phenomenon very likely reflects the *in vivo* immunological communication between T cells and non-T cells and therefore may be a step in the process by which T cells regulate immune responses.[16,17] The cells responding in this reaction show immunological memory and specificities and exert a variety of effector functions including cytotoxic, helper, and suppressor effects.[40]

AMLR was reported to be impaired in patients with diseases characterised by the occurrence of hypergammaglobulinaemia and autoantibodies; a defective suppressor T-cell function has been implicated as the underlying immunopathogenetic mechanism accounting for their development. These diseases include systemic lupus erythematosus, Sjögren's syndrome, infectious mononucleosis etc.[40] The result obtained in this study showed that while the proliferative capability of T cells to autologous B cells' stimulation of ARF patients was lower than that of normal subjects ($p < 0.02$), no such difference was found for other groups of patients when compared to normal subjects. However, the impaired proliferative capability returned to normal six months after their recovery from acute attacks. The impairment of AMLR provides another piece of evidence of defective T-cell function in ARF, although the capability in presenting the Ia-like antigens of B cells of ARF patients had not been studied.

In summary, the altered T-cell functions in terms of decreased numbers of active T, total T, OKT3 and OKT8 cells, loss of suppressor cell activity in the *in vitro* immunoglobulin biosynthesis, diminished PHA response and impaired AMLR may explain partly the pathologically augmented humoral immune response in patients with ARF.

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